REMARKS/ARGUMENTS

The Pending Claims

Claims 86, 88, 89, 92-94, 97, 100, 105, and 107 are pending, and are directed to methods to detect the taxonomic unit of any species of enterobacteria , but no species of another taxonomic unit.

The Amendments to the Claims

The claims have been amended to point out more particularly and claim more distinctly the present invention. In particular, claims 86, 92, and 105 have been amended to recite detection of DNA of any species of the taxonomic unit of Enterobacteriaceae, and no species of another taxonomic unit. This amendment is supported by the specification at, e.g., page 10, lines 19-23, page 25, lines 30-34, page 27, lines 15-22, and page 35, lines 9-24. Step (a) of each of claims 86, 92, 100, and 105 has been amended to recite that the nucleic acid molecules *consist of* at least one or a combination of SEQ ID NO: 2 and SEQ ID NO: 78. Each of claims 86, 92, 100, and 105 also has been amended to recite nucleic acid molecules which exhibit at least 90% identity with SEQ ID NO: 2 and/or SEQ ID NO: 78. This amendment is supported by the specification at, e.g., page 5, lines 20-21, and page 29, lines 27-28. Claims 86, 92, 100, and 105 have further been amended to delete reference to nucleic acid molecules which hybridize specifically with SEQ ID NO: 2 and/or SEQ ID NO: 78. Claims 86 and 100 have been amended to recite, as a method step, that any species of the taxonomic unit of Enterobacteriaceae, and no species of another taxonomic unit, is detected by the formation of the hybrid nucleic acids. This amendment is supported by the specification at, e.g., page 10, lines 19-23, page 25, lines 30-34, page 27, lines 15-22, and page 35, lines 9-24. Claims 92 and 105 have been amended to delete the term "genera." Certain claims also have been amended to correct grammar or matters of form. Claims 96 and 102 have been cancelled. Accordingly, no new matter has been added by way of these amendments.

The Office Action

The Office Action rejects claims 86, 88, 89, 92, 93, 94, 96, 97, 100, 102, 105, and 107 under 35 U.S.C. § 112, first paragraph, as allegedly lacking written description. The Office

Action rejects claims 86, 88, 89, 92-94, 96-98, 102, 105, and 107 under 35 U.S.C. § 103 (a) as allegedly unpatentable over U.S. Patent 5,654,141 (Mariani et al.) ("the Mariani patent") in view of Yamamoto et al. (GenBank Accession Number AB001341, submitted January 25, 1997). Reconsideration of these rejections is hereby requested.

Discussion of Rejection Under 35 U.S.C. § 112, First Paragraph

The Office Action rejects claims 86, 88, 89, 92, 93, 94, 96, 97, 100, 102, 105, and 107 under Section 112, first paragraph, as allegedly lacking written description. In essence, the Office Action argues that the present application does not adequately describe the genus of nucleic acid sequences that exhibit 70% identity or 90% identity to SEQ ID NO: 2 and SEQ ID NO: 78. This rejection is traversed for the reasons set forth below.

Applicants note that claims 86, 92, 100, and 105 have been amended to recite nucleic acid molecules which exhibit at least 90% identity with SEQ ID NO: 2 and/or SEQ ID NO: 78. According to the Office Action, the present application expressly describes only SEQ ID NO: 2 and SEQ ID NO: 78, and not the "hundreds" of nucleic acid sequences that are complements of or have 70% or 90% identity to SEQ ID NO: 2 and/or SEQ ID NO: 78. Thus, the Office Action concludes that the specification does not disclose a representative number of species of the claimed genus.

Contrary to the assertion of the Office Action, the genus of nucleic acid sequences that are 90% identical to SEQ ID NO: 2 does not contain "hundreds" of species. In this respect, SEQ ID NO: 2 is 20 nucleotides in length. A sequence that is 90% identical to a 20 nucleotide sequence can contain, at most, 2 mismatched nucleotides. Using a stochastic calculation for determining combinations, Applicants determined that the number of nucleic acid molecules sharing 90% identity with a 20 nucleotide target sequence such as SEQ ID NO: 2 is only 190. Accordingly, the genus of nucleic acid molecules encompassed by SEQ ID NO: 2 is not as large as alleged by the Office Action.

The written description requirement is satisfied by the disclosure of sufficiently detailed, relevant identifying characteristics of a chemical or biological material, i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and

structure, or some combination of such characteristics. See *Enzo Biochem, Inc. v. Gen-Probe, Inc.*, 323 F.3d 956, 969-70, 63 U.S.P.Q. 2d 1609, 1617 (Fed. Cir. 2002). At the time the subject application was filed, it was well known that primer sequences of 20 nucleotides (e.g., SEQ ID NO: 2) to 30 nucleotides (e.g., SEQ ID NO: 78) could be modified so as to generate variant sequences of similar efficiency. For example, Christopherson et al., *Nucleic Acid Research*, 25: 654-658 (1997) (enclosed herewith), discloses the effects of internal primer template mismatches on the efficiency of reverse transcription and PCR amplification. As outlined in the abstract, the presence of two to four mismatches in a sequence of 30 nucleotides (i.e., sequences that share 93% and 86% identity, respectively, with the normal primer) in primer template duplexes does not have a significant effect on RT-PCR. However, the presence of five or six mismatches (i.e., sequences that share less than 85% identity with the normal primer) reduces the PCR product yield by 22 to 100-fold, respectively, relative to the homogenous template.

Accordingly, as evidenced by the Christopherson reference, nucleic acid sequences that share at least 90% identity with SEQ ID NO: 2 and/or SEQ ID NO: 78 are capable of amplifying DNA targets with the same efficiency as SEQ ID NO: 2 and/or SEQ ID NO: 78. Given the disclosed structure of SEQ ID NOs: 2 and 78 coupled with the disclosed function, one of ordinary skill in the art reading the present application would have been readily apprised of the possible variations in the sequence of SEQ ID NOs: 2 and 78 that would yield a nucleic acid molecule with at least 90% sequence identity to SEQ ID NOs: 2 and 78 and retain the functional characteristics SEQ ID NOs: 2 and 78. In other words, one of ordinary skill in the art would have understood that Applicants had possession of nucleic acid sequences that have at least 90% identity to a nucleic acid molecule consisting of SEQ ID NO: 2 and/or SEQ ID NO: 78. Thus, the written description requirement is satisfied with respect to claims 86, 92, 100, and 105.

This situation is in distinct contrast with *The Regents of the University of California vs. Eli Lilly & Co.* 43 U.S.P.Q.2d 1398 (Fed. Cir. 1997) (see Office Action at page 3). Based on the disclosure of the subject application and the knowledge in the art, one of ordinary skill in the art would appreciate not only the function of sequences at least 90% identical to SEQ ID NO: 2 and/or SEQ ID NO: 78, but also the structure necessary to obtain the desired amplification efficiency. That is, one of ordinary skill in the art would know that modifying

only two of the 20 nucleotides of SEQ ID NO: 2 and/or 3 of the nucleotides of SEQ ID NO: 78 would result in nucleic acid sequences that are at least 90% identical to SEQ ID NO: 2 and/or SEQ ID NO: 78.

In view of the foregoing, the Section 112, first paragraph, rejection of the claims as lacking adequate written description should be withdrawn.

Discussion of Rejection Under 35 U.S.C. § 103(a)

The Office Action rejects claims 86, 88, 89, 92-94, 96-98, 102, 105, and 107 under Section 103(a) as allegedly unpatentable over the Mariani patent in view of Yamamoto et al. The Office Action also relies upon Buck et al., *BioTechniques*, 27: 528-536 (1999), in support of the obviousness rejection. This rejection is traversed for the reasons set forth below.

Specifically, the Office Action alleges that the Mariani patent discloses certain features of a method for detecting bacteria in an analyzed sample as claimed in claims 86, 88, 89, 92-94, 97, 105, and 107. The Office Action concedes that the Mariani patent does not disclose SEQ ID NO: 2. The Office Action further alleges that Yamamoto et al. discloses SEQ ID NO: 2. The Office Action moreover relies on the teachings of Buck et al. to demonstrate that one of ordinary skill in the art would have a reasonable expectation of success with the primers allegedly disclosed by Yamamoto et al. for use in the method of detecting bacteria in an analytical sample, which method is purportedly disclosed by the Mariani patent.

Step (a) of each of claims 86, 92, 100, and 105 has been amended to recite that the nucleic acid molecules *consist of* at least one or a combination of SEQ ID NO: 2 and SEQ ID NO: 78. Claims 96 and 102 have been cancelled. Thus, the rejection as it pertains to claims 96 and 102 is moot.

The Office Action on page 5 states that the Mariani patent allegedly teaches a method of detecting enterobacteria in an analytical sample, since the Mariani patent allegedly teaches a species of enterobacteria, namely, *E. coli* (column 2, lines 52-54, and example 1). However, the Mariani patent does not teach a method of detecting any species of the taxonomic unit of enterobacteria and no other taxonomic unit. On the contrary, the Mariani

patent discloses a method of detecting *E. coli*, *Streptococci*, *Staphylococci*, and/or *Bacteroides* by using sequences which target the 16S rRNA gene, which gene is conserved among these bacteria, or sequences which target the ial gene of *E. coli*, the enterotoxin A gene of *Staphylococcus*, or the non-conserved region of the 16S gene of *Bacteroide fragilis*. *Streptococci*, *Staphylococci*, and *Bacteroides*, however, are not enterobacteria. Thus, the method of the Mariani patent, in which sequences which target the 16S rRNA gene are used, would detect bacteria other than enterobacteria, and the method of the Mariani patent, in which sequences which target the ial gene of *E. coli* are used, would not necessarily detect other enterobacterial species, such as *Yersinia aldovae*. Therefore, the Mariani patent does not teach the presently claimed methods.

None of the cited references, including Yamamoto et al., teaches or suggests the use of a nucleic acid molecule *consisting of* SEQ ID NO: 2 or SEQ ID NO: 78, let alone a combination of nucleic acid molecules *consisting of* SEQ ID NOs: 2 and 78, or a combination of nucleic acid molecules as otherwise recited in the pending claims. Indeed, as stated in the "Reply to Office Action" dated September 16, 2005, the sequence of Yamamoto et al. is 16,446 base pairs in length, whereas SEQ ID NOs: 2 and 78 are 20 and 30 basepairs, respectively. Neither Yamamoto et al. nor the Mariani patent, either alone or in combination, discloses or suggests a particular region of the sequence of Yamamoto et al. which could be used in a method of detecting a bacteria, e.g., an entire taxonomic unit of enterobacteria, in an analytical sample, let alone teach that SEQ ID NO: 2 or SEQ ID NO: 78, or other nucleic acid molecules recited in the pending claims can be used in such a method.

In view of the foregoing, the combined disclosures of the Mariani patent and Yamamoto et al. do not disclose or suggest the subject matter of the pending claims. As such, the obviousness rejection cannot stand and should be withdrawn.

Conclusion

Applicants respectfully submit that the patent application is in condition for allowance. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned agent.

Application No. 10/088,966

Reply to Office Action

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Date: August 14, 2006

654–658 Nucleic Acids Research, 1997, Vol. 25, No. 3

The effects of internal primer–template mismatches on RT-PCR: HIV-1 model studies

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Received August 2, 1996; Revised and Accepted November 22, 1996

ABSTRACT

We investigated the effects of internal primer–template mismatches on the efficiency of reverse transcription and PCR amplification. As models, RNA transcripts representative of different HIV-1 group M subtypes were evaluated with a previously described *gag* primer pair system. We observed that the presence of two to four mismatches in the primer–template duplexes did not have a significant effect on RT-PCR. However, the presence of five and six mismatches with the 28 and 30 base primers reduced PCR product yield by ~22- and 100-fold respectively, relative to the homologous template. The amount of reduction was reproducible from experiment to experiment and was independent of the initial copy number input. Under the conditions used, viral RNA measurements of the more divergent HIV-1 subtypes (A and E) would be underestimated, while isolates of subtypes B, C, D and F–H are expected to be efficiently amplified and accurately measured. The reduced amplification efficiency for targets similar to HIV subtypes A and E can be improved 4- to 10-fold by lowering the annealing temperature and implementing a reverse transcription step that gradually increases in temperature. The additional substitution of either 5-methylcytosine for cytosine throughout or the substitution of inosine at positions of variable bases resulted in a <4-fold difference in product yield between the homologous and most divergent templates.

INTRODUCTION

Nucleic acid-based assays such as the polymerase chain reaction (PCR), the ligase chain reaction (LCR), nucleic acid sequence-based amplification (NASBA) and branched chain DNA (bDNA) rely on the efficient hybridization of oligonucleotides to the targeted sequence. Mismatches between the oligonucleotides and the targeted nucleic acid can affect duplex stability and may compromise the ability of a system to amplify and/or detect the targeted sequence. The effects of mismatches depend on numerous factors, including the length of the oligonucleotide, the nature and position of the mismatches, the temperature of hybridization, the presence of co-solvents and the concentrations of oligonucleotides as well as monovalent and divalent cations (1).

The sequence heterogeneity of human immunodeficiency virus type 1 (HIV-1) challenges efficient detection with nucleic acid-based assays. HIV-1 is divided into groups M and O (2–5). There are presently eight known subtypes within Group M, designated A–H. Subtype A is predominant in Central Africa, B in North America, Europe, South America and Thailand, C in South Africa and India, D in Central Africa, E in Thailand, India and Central Africa and subtypes F–H have been reported in Central Africa. Group O, not yet divided into subtypes, is considered to be more divergent than Group M and to date has been found in a few infected individuals from Cameroon, Gabon and France (4,5). In the US, only six non-B subtypes have been reported so far; one subtype D isolate from a Zairian student (6) and three subtype E, one subtype D and one subtype A from five US servicemen (7).

Using HIV-1 as a model system, we previously reported on the effects of single 3′-terminal mismatches on PCR product yield (8). We found that primers that terminated in a T allowed significant amplification even when mismatched with C, G or T. In this study we systematically examined the effects of multiple internal primer–template mismatches on RT-PCR using templates that represent various HIV-1 subtypes. The amount of product generated with each template was determined with a quantitative RT-PCR assay (9).

MATERIALS AND METHODS

Model system

HIV-1 primers SK462, d(AGTTGGAGGACATCAAGCAGCCATGCAAAT), and SK431, d(TGCTATGTCAGTTCCCCTTGGTTCTCT), and probe SK102, d(GAGACCATCAATGAGGAAGCTGCAGAATGGGAT), were used in this study. The primers were specifically designed to be longer than usual in order to better accommodate mismatches. These primers amplify a 142 bp HIV-1 *gag* region which is highly conserved among the subtype B isolates. However, in non-subtype B isolates, as many as six mutations have been observed in the upstream primer binding region of subtype A isolates and five in the downstream primer binding region of subtype F. To systematically evaluate the effect of mismatches on RT-PCR, a series of templates were constructed that harbored mutations representative of different subtypes (Fig. 1). The templates (designated M1–M8) were engineered to contain mismatches to either the SK462 (M1–M4) or SK431 (M5–M8) primer binding regions; the internal sequences, including the probe binding region were identical in all constructs. A template that is completely homologous to both SK462 and

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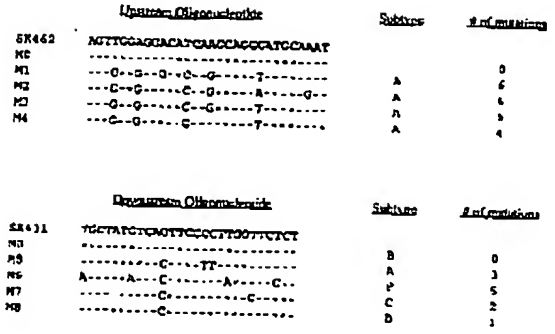

Figure 1. Alignment of the upstream and downstream primers to representative model templates.

SK431 (M0) served as the control. The templates were further engineered to have common sequences flanking the 5′- and 3′-termini. The sequences at the 5′- and 3′-termini encode the T7 promoter transcription signal and a poly(A) tail respectively. The common sequences served two purposes. First, RNA transcripts were generated directly from the amplified products, which eliminated the need to subclone the products into a transcription vector. Second, primers to the common sequences were used to amplify and normalize the templates for subsequent analyses. Different derivatives of SK462 and SK431 were also examined. These derivatives included different base substitutions that either reduced the number of mismatches (as in SK145) or contained different modified bases, such as inosine, 5-methylcytosine, propynyl dU or propynyl dC (15), to increase primer–template stability.

Construction of mutant and control templates

The mutant and control templates were generated by amplification of an HIV-1-containing recombinant plasmid, pSYC1857 (10), with mutagenic primers. Two sets of primers were used to construct the templates (see Fig. 2). To generate templates with various mutations in the SK462 region, common primer D was coupled with mutagenic C primers for the first round of amplification. The second round of amplification was performed with common primers A and B. To generate templates with mutations in the SK431 region, mutagenic D primers were coupled with common primer C for the first round of amplification and common primers A and B for the second round of amplification. Primer A overlaps with primer C by 18 bases; primer B overlaps primer D by 17 bases. Mutations in the SK462 and SK431 regions were selected based on known mutations found in different HIV-1 subtypes (4). A control template that is completely homologous to both primers was similarly constructed.

To construct the templates, 10^4 copies of plasmid pSYC1857 were amplified with 50 pmol of the first set of primers under conditions that minimize nucleotide misincorporation. Specifically, 100 µM each of dATP, dGTP, dCTP and dTTP, 1.5 mM $MgCl_2$ and 2.5 U AmpliTaq DNA polymerase (Perkin Elmer) were used. Stringent cycling conditions were also used: 95°C for 10 s, 50 or 60°C for 10 s and 72°C for 10 s for 30 cycles. The product from the first round was purified by gel electrophoresis and 50 µl of a 10^6 dilution of the product from the first round of amplification

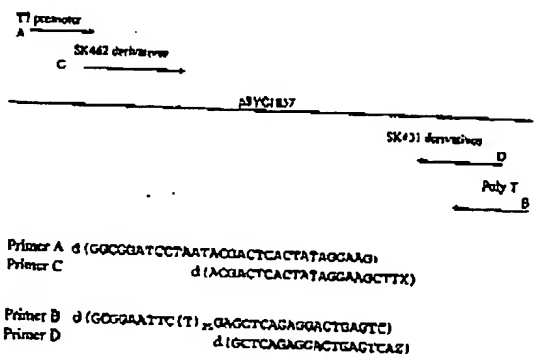

Figure 2. Relative position and sequences of the primers used to generate the different templates. In primer C, X represents either SK462, M1, M2, M3 or M4 (Fig. 1). In primer D, X represents SK431, M5, M6, M7 or M8.

was reamplified with 50 µM of the second set of primers under the high fidelity conditions listed above. In order to generate templates that mimic the native sequence, dTTP rather than dUTP was used.

RNA transcripts

RNA transcripts were prepared directly from the amplified products using a MEGAscript™ transcription kit (Ambion, Austin, TX). Residual DNA was removed from the RNA transcripts by treating with DNase. Proteins were removed by extraction with phenol:chloroform and the RNA was precipitated with ethanol. After resuspension, the transcripts were purified over an Oligotex-dT™ column (Qiagen) as recommended by the manufacturer.

Normalization of templates

The RNA transcripts were normalized to ensure that the same copy number of each test template was used in the evaluation of primer–template mismatches. To normalize the input, the templates were amplified with primers that were completely homologous to all eight templates. Following amplification, the products were quantified on microwell plates coated with SK102 probe as previously described (9). Either 10^4 or 10^5 copies of each template were subsequently used in the mismatch study.

Quantification of the effects of mismatches

To test the effect of mismatches on RT-PCR, the standard cycling conditions recommended for this primer pair were first used: 2 min at 50°C (for uracil N-glycosylase cleavage of any potential carryover of dUMP-containing PCR product from previous reactions) (11); 60°C for 30 min (for reverse transcription); four cycles consisting of a denaturation step (95°C) for 10 s, annealing (50 or 55°C) for 10 s and extension (72°C) for 10 s; followed by 24 cycles of 90, 60 and 72°C for 10 s each. Amplified products were serially diluted and quantified on microwell plates coated with bovine serum albumin-conjugated probe SK102 as previously described (9). Modifications to the standard cycling conditions were subsequently tested. These included lowering the annealing temperature to 50°C and introducing a gradual 'ramp-up' RT step (50–55°C for 5 min, 55–60°C for 15 min, followed by 60°C for 10 min).

Confirmation of template sequence

To ensure that the synthetic templates harbored the expected mutations, each template was purified by gel electrophoresis, cloned into a plasmid vector and the sequence was verified using a Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems).

RESULTS

SK462–431 under standard RT-PCR conditions

Under the standard RT-PCR conditions, the presence of up to four mismatches in either primer had little or no effect on PCR product yield (Table 1). The presence of five mutations in SK462 reduced PCR product yield by 22-fold relative to the homologous template; six mutations by 80- to 100-fold. In SK431, the largest difference (13-fold reduction) was observed with the template that had five mutations. Mismatch tolerance was not affected by the initial copy number input; the differences observed were identical regardless of whether 10^3 or 10^6 copies of the template were amplified (data not shown). Furthermore, the differences were consistent from run to run.

Table 1. The effect of different mutations on PCR product yield

Template	No. of mutations		Difference relative to homologous template	
	SK462	SK431	Standard conditions	Modified conditions
M0	0	0		
M1	6	0	108	10.3
M2	6	0	79	9.9
M3	5	0	22	4.3
M4	4	0	2	1.4
M5	0	3	1.5	1.7
M6	0	5	13	4.3
M7	0	2	0.9	0.6
M8	0	1	1.9	1.6

The templates were amplified under standard and modified conditions as described in Materials and Methods. The results are expressed as fold differences relative to the homologous template (M0).

Effect of reducing the reverse transcription and annealing temperatures on mismatch tolerance

Since the presence of a large number of mutations in either primer affects amplification, two different approaches were taken to alleviate the reduced efficiency. First, the annealing temperature during PCR was reduced from 55 to 50°C to decrease the hybridization stringency. Second, to enhance hybridization, the temperature of the reverse transcription reaction was slowly raised from 50 to 60°C, as described in Materials and Methods. Lowering the annealing temperature to 50°C for the entire 28 cycles allowed for greater tolerance of the mismatches, particularly in those templates that contained mutations in the SK462 binding region. However, the lower annealing temperature by itself was insufficient, as a 9-fold (versus 13-fold) reduction in yield was observed with the five mutations to SK431 (data not shown). When both a 'ramp-up' reverse transcription and a 50°C annealing temperature were applied to all templates, the effect of

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SK462     AGTTGGAGGACATCAAGCAGCCATGCAAAT

Ino 1     ---I--I-----I--I-----I-----I--
Ino 2     ----------I--I-----I-----I--
PC        ----------X--X---X--XX---X----
PU        --ZZ-------Z----------Z-----Z
SK145     ---G--G----------------------
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Figure 3. Alignment of the primer SK462 and its derivatives. I, inosine; X, propynyl C; Z, propynyl U.

mutations was substantially reduced in all of the templates (Table 1). The largest difference was a 10-fold reduction in PCR product with the templates that contained six mutations in the SK462 region, while a 4-fold reduction was seen with the template that contained five mutations in the SK431 binding region.

Alternate primers

Given the adverse effects of multiple mismatches, we further investigated whether modified bases would allow greater mismatch tolerance. Base analogs such as 5-methylcytosine (5-MeC) have been shown to influence not only the binding equilibrium but also increase the dissociation temperature (T_d) of oligonucleotides by changing the base stacking pattern that permits intimate contact between the halogen atom and the adjacent base (12,13). Modified versions of SK462 and SK431 were synthesized containing 5-MeC in place of cytosine (Fig. 3). By using a 50°C annealing temperature, mismatch tolerance was significantly improved. Less than a 5-fold reduction in PCR product yield was observed in templates with the most mismatches (data not shown). With the exception of one position in SK462, the 5-MeC substitutions were not at positions of mismatches.

Derivatives of SK462 and SK431 were synthesized in which the variable bases were replaced with deoxyinosine. Deoxyinosine preferentially pairs with dC and to a lesser extent dA (14). Two inosine-containing derivatives of SK462 were tested. In SK462 Ino 1, six of the variable positions were replaced with inosine, four of which resulted in a dI:dC pairing (Fig. 3). SK462 Ino 2 is similar to SK462 Ino 1 but has two fewer inosine substitutions at the 5′-terminus. Derivatives of SK462 that contained propynyl dC and dU anologs were also evaluated (Fig. 3). Propynyl analogs have been shown to enhance binding of oligonucleotides to an RNA sequence (15).

Since the inosine substitutions were made only to the SK462 primer, only templates M0–M4 were evaluated. Reverse transcription and PCR were performed under the standard conditions. The results indicate that mismatches were better accommodated by SK462 Ino 1, which had inosine substituted at six positions, than by SK462 Ino 2, with four substitutions (Table 2). A <2-fold difference in product yield was detected between the homologous and most divergent templates. Even with inosine substituted at four positions, amplification efficiency with the variant templates was markedly improved. The base pairing of dI with dC and dI with dA likely accounts for the improved mismatch tolerance by creating a more stable duplex. The propynyl derivatives did not perform as well as inosine in accommodating mismatches.

A derivative of SK462, SK145, was also evaluated. Oligonucleotide SK145 differs from SK462 at two positions (Fig. 3) and has fewer mismatches at the 5′-terminus with the majority of isolates. SK462 was designed to eliminate the six consecutive dG nucleotides in SK145 to allow more efficient oligonucleotide

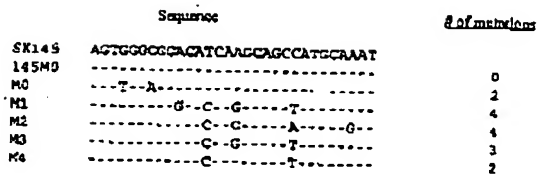

Figure 4. Alignment of primer SK145 with test templates. A homologous template (SK145M0) served as the control template.

synthesis and to better accommodate African isolates such as HIVMAL (16). Unfortunately, subsequent sequence data indicated that the majority of African isolates have a sequence that is homologous to SK145 at the 5′-terminus. Consequently, the two changes in SK462 resulted in a minimum of two mismatches with most HIV-1 isolates. The maximum number of mismatches between SK145 and the known group M subtypes is four. A template that was completely homologous to SK145 was constructed as a control for this study. An alignment of SK145 with the templates is shown in Figure 4. Replacing SK462 with SK145 significantly improved amplification efficiency. Using the 55°C annealing step, a <3-fold reduction in product yield was observed with all the templates (Table 3). These data are consistent with the observation that four mismatches in the SK462 region did not significantly affect product yield. The extent of the differences was similar when the 55°C anneal step was reduced to 50°C, indicating that the lower temperature was not necessary.

Table 2. The effect of modified bases in the upstream primer on product yield

Template	No. of mutations		Differences relative to homologous template				
	SK462	SK431	SK462	SK462 Ino 1	SK462 Ino 2	SK462 propynyl U	SK462 propynyl C
M0	0	0					
M1	6	0	108	0.5	4.0	108	35
M2	6	0	70	1.0	1.3	274	28
M3	3	0	22	1.3	2.7	16	7.3
M4	4	0	2	1.2	5.4	1.0	2.7

Templates were amplified under the standard conditions as described in Materials and Methods.

DISCUSSION

The effect of 3′-terminal mismatches has been extensively reported (17–20). In this study we systematically evaluated the effects of varying numbers of internal mismatches on RT-PCR. The extent of mismatch tolerance is dependent on numerous factors, including the number and position of mismatches, the nature of the mismatches, the length (which influences the T_m value) of the oligonucleotides and the stringency of the amplification conditions used. Mismatches between the template and the reverse transcribing (downstream) primer should affect only the reverse transcription step; mismatches with the upstream primer are expected to affect only the first cycle of amplification. Once a mismatched template is copied, the primers are fully complementary to the PCR products in all subsequent cycles. The more stable RNA–DNA heteroduplexes formed by hybridization of the reverse transcribing primer to RNA templates is expected to allow even greater mismatch tolerance. In the HIV-1 primer pair system studied, as many as four mismatches were readily accommodated and had no effect on RT-PCR under the amplification conditions employed. With five and six mismatches, PCR product yield was reduced 10- to 20-fold and 100-fold respectively.

Table 3. The effect of SK145 on product yield

Template	No. of mutations to SK145	Difference relative to 145M0
145M0	0	
M0	2	0.9
M1	4	1.4
M2	4	2.7
M3	3	2.5
M4	2	1.3

The templates were amplified under the standard conditions used for the assay.

To improve amplification of mismatched templates, numerous changes can be implemented. In this study we examined the effect of different annealing temperatures and various modified bases in the primers on mismatch tolerance. A comparison of the results from 50 and 55°C annealing indicates that mismatches can be better accommodated when a lower annealing temperature is used. Modifications in the reverse transcription step also improved mismatch tolerance in the RT primer. The gradual temperature increase presumably facilitates the priming and extension of mismatched templates. The substitution of modified bases such as 5-meC further stabilized binding of the primer to the template. We have also examined the effect of mismatches on probe hybridization and found that five mismatches (presently found only in some group O isolates) had no effect on detection under the hybridization conditions used for the microwell plate system (data not shown).

Quantitative assays have been extensively used in monitoring the effect of anti-retroviral drugs and for use as a prognostic indicator (21–24). The clinical efficacy of these drugs was established based upon the ability of the drugs to significantly reduce viral load as determined by quantitative RNA assays. Given the heterogeneity of the HIV genome, we embarked on this study to determine the extent to which primer–template mismatches can affect amplification efficiency. The templates used in this study were intentionally constructed to represent worst case scenarios. For example, mutant templates of SK462 represent the most divergent of HIV isolates, the A subtypes. The downstream reverse transcribing primer binding region, SK431, is more conserved, with a maximum of five mismatches to a subtype F isolate.

We infer from the results of this study that isolates of subtype B, which are predominant in the US and Europe, will be efficiently amplified and detected given that they harbor at most three mismatches with SK462 and two mismatches with SK431. Similarly, isolates of subtypes C and D are expected to be efficiently amplified. On the other hand, the viral RNA titers of some subtype A isolates will be underestimated due to the presence of as many as six mismatches to the SK462 region.

Subtype E isolates, although distinct from subtype A over the *env* region, are indistinguishable from subtype A in the *gag* gene (3). Consequently, amplification of some subtype E isolates will also be underestimated. The number of available sequences for subtypes F–H are limited, but based on the sequences available to date, are expected to be amplified efficiently with SK462-431. It is important to note that although the absolute copy number determinations for subtypes A and E may be compromised, the reduction in amplification efficiency was consistent from experiment to experiment, suggesting that the assay can still be used to monitor viral load in an individual over time. These studies indicate that replacing SK462 with SK145 would significantly improve quantification of the more divergent isolates. Furthermore, the incorporation of modified bases into the primers would further enhance mismatch tolerance.

This study extends earlier work on the effects of primer–template mismatches on PCR amplification. We have demonstrated that mismatch tolerance can be improved by reducing the number of primer–template mismatches, reducing the annealing temperature, using a slow temperature ramp during reverse transcription and using modified bases to minimize destabilization of mismatches. The results of this experimental study should serve as guidelines in the design of primers for other systems. Our future plans include extending these studies to clinical specimens of known HIV-1 subtypes.

ACKNOWLEDGEMENTS

We thank Tom White and Tom Myers for critical review of the manuscript, Sheng-Yung Chang for technical advice, Fred Reichert and Agnes Cavalli for sequencing the cloned templates, Corey Levenson, Tomas Martinez, Laura Jung and Olga Budker for providing oligonucleotides and Annie Yoon for manuscript preparation.

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